bio-protocol Preprint

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Ribosome profiling with retapamulin treatment (Ribo-RET)

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An abbreviated version of this protocol was published in eLIFE

Pervasive translation in *Mycobacterium tuberculosis*

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Detailed protocol

RIBORET PROTOCOL – Mycobacterium smegmatis (Msmeg) or Mycobacterium tuberculosis (Mtb)

I. Grow cells/collect and mill

Make lysis buffer:
20 mM Tris pH 8.0
10 mM $MgCl_2$
100 mM NH_4Cl
5 mM $CaCl_2$
0.4% Triton X100
0.1% NP-40
1 mM chloramphenicol

Add just before filter: 100 U/mL DNase I

Grow cells to OD_{600} = 1.0 for Msmeg and Mtb
Add Retapamulin 0.125 mg/mL. Incubate 15 min at room temp with occasional swirling.

Add DNase I to 700 uL lysis buffer

Fill 50 mL conical with liquid N_2 (to ~40 mL mark) and add 700 uL [lysis buffer + DNase I] dropwise.
Scrape frozen [lysis buffer + DNase I] from sides of conical.
Filter cells using 0.22 um filter. Scrape cells and dip cell scraper in liquid N_2. May need to use spatula to get cells off of scraper.
Use heated needle to make ~ 4 holes in cap to 50 mL conical. Cap conical and store at -80°C.

Mill cells:
6 cycles for Msmeg, 8 cycles for Mtb
Each cycle 3 minutes, frequency 15.
Put cups in liquid N_2 to re-freeze between each cycle.

Fill conical to ~ 40 mL mark with liquid N_2. Dip spatula in liquid N_2 to chill spatula. Transfer milled cells to 50 mL conical containing liquid N_2. Use a heated needle to make ~ 4 holes in cap to 50 mL conical. Cap conical and store at -80°C.

NOTE: if powder is yellow, cells were not ground efficiently and will need to mill for more cycles.

I. Total RNA Isolation and Sample Extraction for Monosome Purification

A. Total RNA Isolation (RNA-seq samples)

Make RNA lysis buffer = 14.4 mL DEPC H_2O
300 uL 3 M Na Acetate
250 uL 0.5 M EDTA

Warm 0.7 mL acid phenol:chloroform to 65°C

Add 0.6 mL RNA Lysis buffer + 2 heaping loopfuls of milled cells to microcentrifuge tube. Invert to mix.
[See B. with what to do with rest of milled cells.]

Add 80 uL 10% SDS to tube.
Add 0.7 mL acid phenol:chloroform (warmed to 65°C). Wrap tube in parafilm. Vortex.
Incubate 5' at 65°C
Incubate 5' on ice
Spin 5 min at max speed (~15,000 rpm)
Transfer upper phase to new tube containing 0.7 mL acid phenol:chloroform (at RT). Vortex.
Incubate 5' at RT
Spin 5 min max speed
Transfer upper phase to new tube containing 0.7 mL chloroform. Vortex.
Spin 5 min max speed
Transfer upper phase to new tube, measure volume. Add 0.1V 3 M Na Acetate + 1V isopropanol. Invert to mix. Precipitate at -80°C for 3 hours min or overnight.

Spin 30' at max speed (~15,000 rpm) at 4°C. Pipet off supernatant. Add 0.7 ml 70% EtOH (DEPC H_2O) to side of tube away from pellet (do not disturb pellet) and spin 10' at max speed at 4°C. Pipet off supernatant. Repeat for second wash. Dry pellet at RT. Resuspend pellet in 100 uL DEPC H_2O.
NOTE: If suspect that concentration of RNA low, resuspend in lower volume.
If pellet does not go into solution, add another 100uL DEPC H_2O and let sit 10 min at RT. Nanodrop.

Store in -80°C freezer. Will use this RNA for RNA-seq library prep.

A. Rest of powder: use for extract preparation for monosome purification

Thaw powder at 30°C for 2 min. Then leave at RT until thawed.
Incubate 20-30 min on ice.
Transfer all to new 1.7 mL tube, spin 4°C max speed (~15,000 rpm) for 10 min.
Transfer supernatant to new tube.
[For BSL- 2 strains pass through 0.22 um syringe filter.]
Dilute 1 uL supernatant + 99 uL DEPC H_2O and nanodrop.
Calculate volume for 1 mg and set up aliquots of 1 mg RNA. Add lysis buffer (same as used on day 1 for harvesting cells) so final volume = 150 uL. Flash freeze in liquid N_2. Store in -80°C freezer.
Note: if less than 1 mg, set up two aliquots of equal volume. Make sure volumes are equal because will be adding them to ultracentrifuge tubes and will need to balance tubes.
Will use these samples for MNase digest and sucrose gradient.

RNA-seq Library Prep

I. DNase treat RNA and remove ribosomal RNA

A. DNase treat 6-8 ug RNA.

A. Remove ribosomal RNA

Use NEBNext rRNA Depletion Kit to remove ribosomal RNA.

Use 1 ug DNase treated RNA as input.
Follow kit protocol with these modifications:

1.1 Probe Hybridization to RNA
Set up 15 uL reaction. Use mycoprobe specific to Msmeg:
Add 1 ug total RNA (10 uL)
Add 1 uL NEBNext Bacterial rRNA Depletion Solution
Add 2 uL NEBNext Probe Hybrization Buffer
Add 2 uL Mycoprobe

1.2 Follow kit protocol

1.3 Follow kit protocol

1.4 Use NEBNext RNA Sample Purification Beads. Follow kit protocol.

I. Fragment RNA

Make 2x fragmentation buffer

0.5 M EDTA 1 uL
0.1 M Na_2CO_3 30 uL
0.1 M $NaHCO_3$ 220 uL

NOTE: It is recommended to test fragmentation buffer with another RNA sample to determine optimal incubation time.

Transfer RNA to PCR tube (25 uL).
Add 25 uL 2x fragmentation buffer to RNA
Incubate at 95°C for 25 min (or other optimal incubation time)

Isopropanol precipitate:
After incubation at 95°C, transfer 50 uL from PCR tube to 1.7 mL tube.
Add 450 uL DEPC H_2O
50 uL 3 M Na Acetate
2 uL glycogen (20 mg/mL stock)
600 uL isopropanol

Precipitate at -80°C for 3 hours min or overnight.

Spin 30' at max speed (~15,000 rpm) at 4°C. Pipet off supernatant. Add 0.7 ml 70% EtOH (DEPC H_2O) to side of tube away from pellet (do not disturb pellet) and spin 10' at max speed at 4°C. Pipet off supernatant. Repeat for second wash. Dry pellet at RT. Resuspend pellet in 20 uL DEPC H_2O.

Go to section VII. for rest of RNA-seq library prep.

Ribosome Footprinting

I. Sucrose gradient

A. Make sucrose gradient buffer:

Can make sucrose gradient buffer 1 day ahead of time (not earlier). Need 6 mL per sample. Make 10% sucrose buffer and 50% sucrose buffer:

10% or 50% Sucrose
20 mM Tris pH 8.0
10 mM $MgCl_2$
100 mM NH_4Cl
1 mM chloramphenicol
2 mM DTT
DEPC H_2O

Add ~ ½ volume DEPC H_2O
Add sucrose.
Tape to vortex to mix until sucrose in solution. Then add rest of reagents. Bring to desired volume with DEPC H_2O.
Store in refrig o/n.

A. MNase digest prior to running sucrose gradient

Digest sample with 1500 units MNase.
MNase (Roche 10107921001) supplied at 15,000 units/mg. Add 40 uL 50 mM Tris pH 8.0 = 375 U/uL. Set up aliquots and store at -80°C.

MNase digest:
Add to tube (1 mg):
6 uL RNase inhibitor (SUPERase IN)
4 uL enzyme (1500 Units)

Incubate 1 hour at RT on rotisserie. Quench reaction by adding 2 uL 0.5 M EGTA. Invert to mix, spin samples briefly and place on ice.

During incubation, chill ultracentrifuge
Set speed = 35,000 rpm, temp = 4°C, time = 3hrs

A. Spin samples through 10% - 50% sucrose gradient

Make 10% - 50% sucrose gradient.
Pipet MNase digested sample (all) on top of sucrose gradient.

Balance centrifuge tubes.
Balance buffer:
20 mM Tris pH 8.0
10 mM $MgCl_2$
100 mM NH_4Cl
1 mM chloramphenicol
DEPC H_2O

Spin in ultracentrifuge 35,000 rpm, 4°C, 3 hours

I. Fractionation

Collect 400 uL fractions for each gradient.

Load 10 uL of each fraction on 1% agarose gel+1% bleach.
Pick monosome fractions.

Phenol extract/isopropanol precipitate samples with correct band pattern (or can freeze samples at -80°C and set up extraction/precipitation later). Usually are two fractions.
Add 80 uL 10% SDS to monosome solution.
Add 0.7mL acid phenol:chloroform (warmed to 65°C). Wrap tube in parafilm. Vortex.
Incubate 5' at 65°C
Incubate 5' on ice
Spin 5 min max speed
Transfer upper phase to new tube containing 0.7 mL acid phenol:chloroform (at RT). Vortex.
Incubate 5' at RT
Spin 5 min max speed
Transfer upper phase to new tube containing 0.7 mL chloroform. Vortex.
Spin 5 min max speed
Transfer upper phase to new tube, measure volume. Add 0.1V 3 M Na Acetate + 1V isopropanol. Invert to mix. Precipitate at -80°C for 3 hours min or overnight.

Spin 30' at max speed (~15,000 rpm) at 4°C. Pipet off supernatant. Add 0.7 ml 70% EtOH (DEPC H_2O) to side of tube away from pellet (do not disturb pellet) and spin 10' at max speed at 4°C. Pipet off supernatant. Repeat for second wash. Dry pellet at RT. Resuspend pellet in 20 uL DEPC H_2O.
If have two extractions, resuspend first pellet in 20 uL DEPC H_2O then transfer to second tube and resuspend pellet. Nanodrop.

Store in -80°C freezer.

For RNA-seq and Ribosome Profiling Samples (run both samples in parallel)

I. Size selection

Pour 15% denaturing gel.

6 mL urea gel – system concentrate
3 mL urea gel – system diluent
1 mL urea gel – system buffer
100 uL 10% APS
10 uL TEMED

Set up samples to load on gel:

low range ssRNA ladder
~15 nt size marker (15 nt RNA oligo)
samples (for ribosome profiling samples, split into at least two wells on gel to avoid overloading lanes.)

Heat all samples at 80°C, 1400 rpm, 2 min to denature RNA.
Place samples on ice if not ready to load on gel.

Load samples on gel.

Run at 200 V until lower dye marker has just run off gel.
Stain gel with 1 uL EtBr

Cut out gel from 15-50 nt. Using a heated needle, make a hole in the bottom of a 0.5 mL tube. Add gel to tube. If there is too much gel for one tube, set up a second tube. Put 0.5 mL tube inside 1.7 mL tube. Spin 5 min max speed (~15,000 rpm) to shred gel. Add 500 uL RNA gel extraction buffer to 1.7mL tube. Put on nutator in cold room overnight.

RNA gel extraction buffer:
300 mM NaOAc (pH 5.5)
1 mM EDTA
0.1 U/uL SUPERase-IN

am:
Transfer all, including gel pieces, to Spin-X column. Spin 5' max speed (~15,000 rpm). Transfer liquid to new tube. Add: 2 uL glycogen (20 mg/mL stock) + 500 uL isopropanol. Precipitate at -80°C for 3 hours min or overnight.

Spin 30' at max speed (~15,000 rpm) at 4°C. Pipet off supernatant. Add 0.7 ml 70% EtOH (DEPC H_2O) to side of tube away from pellet (do not disturb pellet) and spin 10' at max speed at 4°C. Pipet off supernatant. Repeat for second wash. Dry pellet at RT. Resuspend pellet in 15 uL DEPC H_2O.
If have two extractions, resuspend first pellet in 15 uL DEPC H_2O then transfer to second tube and resuspend pellet.

I. Dephosphorylation

Samples are in 15uL volume. Denature samples 90 s at 80°C. Equilibrate to 37°C.
Add: 2 uL 10X PNK buffer + 1 uL SUPERase IN + 2 uL T4 PNK.
Incubate 37°C for 1 hour, then 4°C.

After 1 hour incubation, add 380 ul DEPC water + 40 uL 3 M Na Acetate + 2 uL glycogen (20 mg/mL). Add 500 ul isopropanol. Precipitate at -80°C for 3 hours min or overnight.

Spin 30' at max speed (~15,000 rpm) at 4°C. Pipet off supernatant. Add 0.7 ml 70% EtOH (DEPC H_2O) to side of tube away from pellet (do not disturb pellet) and spin 10' at max speed at 4°C. Pipet off supernatant. Repeat for second wash. Dry pellet at RT. Resuspend pellet in 8 uL DEPC H_2O.

I. Ligation

Add 2 uL of preadenylated linker (JW9371, 0.5 ug/ul) to 8 uL dephosphorylated RNA. Denature for 90 s at 80°C, and then cool to room temperature.

Set up ligation:

RNA and linker	10.0 uL
10x T4 RNA Ligase Reaction Buffer	2.0 uL
PEG 8000 (50% wt/vol)	6.0 uL – 15% wt/vol

PEG 8000 (50% wt/vol)	6.0 uL	15% wt/vol
SUPERase IN (20 U/ul)	1.0 uL	20 U
T4 RNA Ligase 2, truncated KQ	1.0 uL	200 U

Incubate 16 h at 16°C.

Set up precipitation.
Add:

480 uL DEPC H_2O
2 uL glycogen (20 mg/mL stock)
50 uL 3M Na Acetate
500 uL isopropanol

Precipitate at -80°C for 3 hours min or overnight.

Spin 30' at max speed (~15,000 rpm) at 4°C. Pipet off supernatant. Add 0.7 ml 70% EtOH (DEPC H_2O) to side of tube away from pellet (do not disturb pellet) and spin 10' at max speed at 4°C. Pipet off supernatant. Repeat for second wash. Dry pellet at RT. Resuspend pellet in 10 uL DEPC H_2O.

Note: during this time, pour 15% denaturing gel

Set up samples to load on gel:

low range ssRNA ladder
mock ligation to use as size selector
30 nt size marker (30 nt RNA oligo)
samples

Use loading dye without xylene cyanol because xylene cyanol interferes with visualization of the ligation product.

Heat all samples at 80°C/1400rpm for 3 min. Place on ice if not loading on gel right away.

Load gel. Run at 200V until lower dye marker has just run off gel. Stain gel with 1uL EtBr.

Cut out band at same size as mock ligation (~30 nt to 80 nt).

Using a heated needle, make a hole in the bottom of a 0.5 mL tube. Add gel to tube. Put 0.5 mL tube inside 1.7 mL tube. Spin 5 min max speed (~15,000 rpm) to shred gel. Add 500 uL RNA gel extraction buffer to 1.7mL tube. Put on nutator in cold room overnight.

am:
Transfer all, including gel pieces, to Spin-X column. Spin 5' max speed (~15,000 rpm). Transfer liquid to new tube. Add: 2 uL glycogen (20 mg/mL stock) + 500 uL isopropanol. Precipitate at -80°C for 3 hours min or overnight.

Spin 30' at max speed (~15,000 rpm) at 4°C. Pipet off supernatant. Add 0.7 ml 70% EtOH (DEPC H_2O) to side of tube away from pellet (do not disturb pellet) and spin 10' at max speed at 4°C. Pipet off supernatant. Repeat for second wash. Dry pellet at RT. Resuspend pellet in 10 uL DEPC H_2O.

I. Reverse transcription

Pour 10% denaturing gel:
4 mL urea gel – system concentrate
5 mL urea gel – system diluent
1 mL urea gel – system buffer
100 uL 10% APS
10 uL TEMED

Add 2.0 uL of reverse transcription primer JW8875 at 1.25 uM to ligated RNA. Denature for 2 min at 80 °C in a thermocycler and then place on ice.

Set up RT:

Ligation and primer	12.0 uL
5x First-strand buffer	4.0 uL
10 mM dNTPs	1.0 uL
0.1 M DTT	1.0 uL
SUPERase.In (20 U/uL)	1.0 uL
SuperScript III (200 U/uL)	1.0 uL

Incubate 30 min at 55°C (for high-GC template Mycobacteria) in thermocycler.

Hydrolyze the RNA by adding 2.3 uL 1 N NaOH to each tube

Incubate 95°C 15 min (to inactivate enzyme)
Hold 80°C

Set up samples to load on gel:
low range ssRNA ladder
RT oligo JW8875 (1.25 uM stock)
samples

Use loading dye without xylene cyanol because xylene cyanol interferes with visualization of the bands.

Run at 200V. Run approx 10-15 min after lower dye marker off of gel.
Stain gel with 1uL EtBr

Cut out smear above oligo band. Avoid cutting out oligo band.

Using a heated needle, make a hole in the bottom of a 0.5 mL tube. Add gel to tube. If there is too much gel for one tube, set up a second tube. Put 0.5 mL tube inside 1.7 mL tube. Spin 5 min max speed (~15,000 rpm) to shred gel. Add 500 uL DNA gel extraction buffer to 1.7mL tube. Put on nutator in cold room overnight.

am:
Transfer all, including gel pieces, to Spin-X column. Spin 5' max speed (~15,000 rpm). Transfer liquid to new tube. Add: 2 uL glycogen (20 mg/mL stock) + 500 uL isopropanol. Precipitate at -80°C for 3 hours min or overnight.

Spin 30' at max speed (~15,000 rpm) at 4°C. Pipet off supernatant. Add 0.7 ml 70% EtOH to side of tube away from pellet (do not disturb pellet) and spin 10' at max speed at 4°C. Pipet off supernatant. Repeat for second wash. Dry pellet at RT. Resuspend pellet in 15 uL nuclease free water.
If have two extractions, resuspend first pellet in 15 uL nuclease free water then transfer to second tube and resuspend pellet.

I. Circularization

Transfer samples to PCR tubes.

Use CircLigase kit from Lucigen.

Add to each sample (15 uL):
10x buffer 2 uL
1 mM ATP 1 uL
50 mM $MnCl_2$ 1 uL
CircLigase 1 uL

Incubate in thermocycler 60°C for 1hr 30m then 80°C for 10m

(note: can proceed to optional rRNA removal step before going on to PCR)

If **not** doing optional rRNA removal step, during circ reaction, pour 8% non-denaturing polyacrylamide gel (10 well comb). Will need these for after PCR amplification of library. Will test 4 different cycle numbers per sample.

Mix for 1 gel:	Mix for 2 gels:
7 mL sdH_2O	10.5 mL sdH_2O
1 mL 10x TBE	1.5 mL 10x TBE
2 mL acrylamide	3 mL acrylamide
100 uL 10% APS	150 uL 10% APS
10 uL TEMED	15 uL TEMED

I. PCR amplification of library

Not necessary to clean up sample before PCR.

Master mix – this is good for 4 PCRs. Set up 1 master mix for each sample

Master mix:

16.7 uL 5x Phusion HF buffer (NOT GC)
1.7 uL 10 mM dNTPs
0.8 uL JW8835 (50 uM) forward primer
0.8 uL JW? (50 uM) barcoded reverse primer = I7
5 uL circularized DNA *

58.4 uL sdH_2O
0.8uL Phusion polymerase

*if working with high concentration of DNA, use 2.5 uL of circularized DNA and increase water to 61 uL.

Aliquot 20uL into each of 4 PCR tubes

Run program on thermocycler:
98°C 30 sec
Then x cycles:
98°C 10 sec
60°C 10 sec
72°C 5 sec

Remove PCR tubes and place on ice after designated # of cycles (usually start by testing 4, 6, 8 and 10 cycles).

Add gel loading dye and run all on polyacrylamide gel, 180V for 5 min then 120V for 1 hour.

Stain with 1uL EtBr

Cut out ~180 bp band (above adapter band). If bands weak, cut out band in all lanes.

Using a heated needle, make a hole in the bottom of a 0.5 mL tube. Add gel to tube. Put 0.5 mL tube inside 1.7 mL tube. Spin 5 min max speed (~15,000 rpm) to shred gel. Add 500 uL DNA gel extraction buffer to 1.7mL tube. Put on nutator in cold room overnight.

am:
Transfer all, including gel pieces, to Spin-X column. Spin 5' max speed (~15,000 rpm). Transfer liquid to new tube. Add: 2 uL glycogen (20 mg/mL stock) + 500 uL isopropanol. Precipitate at -80°C for 3 hours min or overnight.

Spin 30' at max speed (~15,000 rpm) at 4°C. Pipet off supernatant. Add 0.7 ml 70% EtOH to side of tube away from pellet (do not disturb pellet) and spin 10' at max speed at 4°C. Pipet off supernatant. Repeat for second wash. Dry pellet at RT. Resuspend pellet in 10 uL nuclease free water

Qubit quantify. If enough DNA, send for sequencing. If not, repeat PCR using optimal number of cycles.

APPENDIX:

Oligo sequences:

3' preadenylated linker JW9371: /5rApp/CTGTAGGCACCATCAAT/3ddC/

Mock ligation JW9370: rArCrArCrUrCrUrUrUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUCTGTAGGCACCATCAAT/3ddc/

RT oligo
JW8875: /5Phos/AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGC/iSp18/CACTCA/iSp18/TTCAGACGTGTGCTCTTCCGATCTA

Forward PCR primer JW8835: AATGATACGGCGACCACCGAGATCTACAC

Reverse PCR prime (includes index):
Index 34: CAAGCAGAAGACGGCATACGAGATGCCATGGTGACTGGAGTTCAGACGTGTGCTCTTCCG

Index 39: CAAGCAGAAGACGGCATACGAGATGTATAGGTGACTGGAGTTCAGACGTGTGCTCTTCCG

Can follow this format and design other indexing primers.

How to cite:(Readers should cite both the Bio-protocol preprint and the original research article where this protocol was used)

1. Derbyshire, K. M., Gray, T. A. and Wade, J. T.(2022). Ribosome profiling with retapamulin treatment (Ribo-RET). Bio-protocol Preprint. bio-protocol.org/prep1719.
2. Canestrari, J. G., Champion, M. M., Derbyshire, K. M., Gray, T. A., Wade, J. T., Wang, A. J. and Smith, C. Pervasive translation in *Mycobacterium tuberculosis*. eLIFE. DOI: 10.7554/eLife.73980